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UTILITY PATENT APPLICATION TRANSMITTAL

(Only for new nonprovisional applications under 37 CFR 1.53(b))

Attorney Docket No,	960296.95360
First Inventor or Application Identifier	Ronald T. Raines
Title	Oxidation-Resistant Ribonuclease Inhibitor
Express Mail Label No.	EJ311815415US

APPLICATION ELEMENTS

See MPEP Chapter 600 concerning utility patent application contents.

ADDRESS TO: **Assistant Commissioner for Patents, Box Patent Application, Washington, D.C. 20231**

1 [X] Fee transmittal Form (Submit an original and a duplicate for fee processing)

2 [X] Specification [Total [18]] *(preferred arrangement set forth below)*
- Descriptive title of the invention
- Cross References to Related Applications
- Statement Regarding Fed Sponsored R&D
- Reference to Microfiche Appendix
- Background of the Invention
- Brief Summary of the Invention
- Brief Description of the Drawings *(if filed)*
- Detailed Description
- Claim(s)
- Abstract of the Disclosure

3 [X] Drawing(s) (35 USC 113) [Total Sheets [5]]

4. Oath or Declaration [Total Pages [2]]

a. [X] Newly executed (original or copy)

b. [ ] Copy from prior Application (37 CFR 1.63(d)) (for continuation/divisional with Box 17 completed)

[Note Box 5 below]

i. [ ] DELETION OF INVENTOR(S) Signed Statement attached deleting inventor(s) named in prior application, see 37 CFR 1.63(d)(2) and 1.33(b).

5 [ ] Incorporation By Reference (useable if Box 4b is checked) The entire disclosure of the prior application from which a copy of the oath or declaration is supplied under Box 4b, is considered as being part of the disclosure of the accompanying application and is hereby incorporated by reference herein.

6. [ ] Microfiche Computer Program *(Appendix)*

7. Nucleotide and/or Amino Acid Sequence Submission *(if applicable, all necessary)*

[X] Computer readable Copy
[X] Paper Copy (identical to computer copy)
[X] Statement Verifying identity of above

ACCOMPANYING APPLICATION PARTS

8 [ ] Assignment Papers (cover sheet & documents)

9 [ ] 37 CFR 3.73(b) Statement [ ] Power of Attorney (where there is an assignee)

10 [ ] English Translation Document *(if applicable)*

11 [ ] Information Disclosure Statement (IDS)/PTO-1449 [ ] Copies of IDS Citations

12 [ ] Preliminary Amendment

13 [X] Return receipt postcard (MPEP 503) *(Should be specifically itemized)*

14 [X] *Small Entity Statement(s) [ ] Statement filed in prior application Status still proper and desired

15 [ ] Certified copy of priority Document(s) *(if foreign priority is claimed)*

16 [ ] Other:

* A new statement is required to pay small entity fees, except where one has been filed in a prior application and is being relied upon

17. If a CONTINUING APPLICATION, *check appropriate box and supply the requisite information:*

[ ] Continuation [ ] Divisional [ ] Continuation-in-part (CIP) of prior application no. ____/____

Prior application information: Examiner: Group/Art Unit:

18. CORRESPONDENCE ADDRESS

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January 20, 1999

Assistant Commissioner of Patents
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Re: Filing New Patent Application

Dear Sir:

Enclosed for filing please find a new patent application entitled:

OXIDATION-RESISTANT RIBONUCLEASE INHIBITOR

by Ronald T. Raines

The undersigned hereby certifies that this document is being deposited with the United States Postal Service today, January 20, 1999, by the "Express Mail" service, utilizing Express Mail label number EJ311815415US, addressed to: Assistant Commissioner for Patents, Box Patent Application, Washington, DC 20231.

Please indicate receipt of this application by returning the attached postcard with the official Patent and Trademark Office receipt and serial number stamped thereon.

Respectfully submitted,

QBMAD/179340

Attorney's Docket No. 960296.95360

Applicant or Patentee: Raines

Serial or Patent No.:

Filed or Issued:

For: OXIDATION-RESISTANT RIBONUCLEASE INHIBITOR

VERIFIED STATEMENT (DECLARATION) CLAIMING SMALL ENTITY STATUS (37 CFR 1.9(f) and 1.27(d)) - NONPROFIT ORGANIZATION

I hereby declare that I am an official empowered to act on behalf of the nonprofit organization identified below:

NAME OF ORGANIZATION: Wisconsin Alumni Research Foundation

ADDRESS OF ORGANIZATION: P.O. Box 7365, Madison, WI 53707-7365

TYPE OF ORGANIZATION

☑ UNIVERSITY OR OTHER INSTITUTION OF HIGHER EDUCATION TAX EXEMPT UNDER INTERNAL REVENUE SERVICE CODE (26 USC 501(a) and 501(c)(3))

☐ NONPROFIT SCIENTIFIC OR EDUCATIONAL UNDER STATUTE OF STATE OF THE UNITED STATES OF AMERICA

(NAME OF STATE)

(CITATION OF STATUTE)

☐ WOULD QUALIFY AS TAX EXEMPT UNDER INTERNAL REVENUE SERVICE CODE (26 USC 501(a) and 501(c)(3)) IF LOCATED IN THE UNITED STATES OF AMERICA

☐ WOULD QUALIFY AS NONPROFIT SCIENTIFIC OR EDUCATIONAL UNDER STATUTE OF STATE OF THE UNITED STATES OF AMERICA IF LOCATED IN THE UNITED STATES OF AMERICA

(NAME OF STATE)

(CITATION OF STATUTE)

I hereby declare that the nonprofit organization identified above qualifies as a nonprofit organization as defined in 37 CFR 1.9(e) for purposes of paying reduced fees under Section 41(a) and (b) of Title 35, United States Code with regard to the invention entitled

OXIDATION-RESISTANT RIBONUCLEASE INHIBITOR

by inventor(s) Raines

described in

☑ the specification filed herewith.

☐ application serial no. , filed .

☐ patent no. , issued .

I hereby declare that rights under contract or law have been conveyed to and remain with the nonprofit organization with regard to the above identified invention.

If the rights held by the nonprofit organization are not exclusive, each individual, concern or organization having rights to the invention is listed below* and no rights to the invention are held by any person, other than the inventor, who would not qualify as an independent inventor under 37 CFR 1.9(c) if that person made the invention, or by any concern which would not qualify as a small business concern under 37 CFR 1.9(d) or a nonprofit organization under 37 CFR 1.9(e).

*NOTE: Separate verified statements are required from each named person, concern or organization having rights to the invention averring to their status as small entities. (37 CFR 1.27).

NAME: _____

ADDRESS: _____

_____

☐ INDIVIDUAL ☐ SMALL BUSINESS CONCERN ☐ NONPROFIT ORGANIZATION

NAME: _____

ADDRESS: _____

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I acknowledge the duty to file, in this application or patent, notification of any change in status resulting in loss of entitlement to small entity status prior to paying, or at the time of paying, the earliest of the issue fee or any maintenance fee due after the date on which status as a small entity is no longer appropriate. (37 CFR 1.28(b))

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application, any patent issuing thereon, or any patent to which this verified statement is directed.

NAME OF PERSON SIGNING Carl E. Gulbrandsen

TITLE IN ORGANIZATION Director of Patents and Licensing

ADDRESS OF PERSON SIGNING Wisconsin Alumni Research Foundation

P.O. Box 7365, Madison, WI 53707-7365

SIGNATURE Carl E. Gulbrandsen Date 1/5/98

Attorney's Docket No. 960296.95360

Applicant or Patentee: Raines

Serial or Patent No.: _____

Filed or Issued: _____

For: OXIDATION-RESISTANT RIBONUCLEASE INHIBITOR

VERIFIED STATEMENT (DECLARATION) CLAIMING SMALL ENTITY STATUS (37 CFR 1.9(f) and 1.27(b)) - INDEPENDENT INVENTOR

As a below named inventor, I hereby declare that I qualify as an independent inventor as defined in 37 CFR 1.9(c) for purposes of paying reduced fees under Section 41(a) and (b) of Title 35, United States Code, to the Patent and Trademark Office with regard to the invention entitled OXIDATION-RESISTANT RIBONUCLEASE INHIBITOR

described in

☑ the specification filed herewith.

☐ application serial no. _____, filed _____.

☐ patent no. _____, issued _____.

I have not assigned, granted, conveyed or licensed and am under no obligation under contract or law to assign, grant, convey or license, any rights in the invention to any person who could not be classified as an independent inventor under 37 CFR 1.9(c) if that person had made the invention, or to any concern which would not qualify as a small business concern under 37 CFR 1.9(d) or a nonprofit organization under 37 CFR 1.9(e).

Each person, concern or organization to which I have assigned, granted, conveyed, or licensed or am under an obligation under contract or law to assign, grant, convey, or license any rights in the invention is listed below:

☐ no such person, concern, or organization

persons, concerns or organizations listed below*

*NOTE: Separate verified statements are required from each named person, concern or organization having rights to the invention averring to their status as small entities. (37 CFR 1.27)

NAME: Wisconsin Alumni Research Foundation

ADDRESS: P.O. Box 7365, Madison, WI 53707-7365

☐ INDIVIDUAL ☐ SMALL BUSINESS CONCERN ☐ NONPROFIT ORGANIZATION

NAME: _____

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I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application, any patent issuing thereon, or any patent to which this verified statement is directed.

Ronald Raines

Signature of inventor

Date 1/11/99

jlh:sment\Raines independent (98182)

OXIDATION-RESISTANT RIBONUCLEASE INHIBITOR

CROSS-REFERENCE TO RELATED APPLICATIONS

Not applicable.

STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH OR DEVELOPMENT

The work described herein was supported in part by NIH Grants GM44783, CA73808 and AR44276, and NSF Grant BES9604563. The federal government may have certain rights in this invention.

BACKGROUND OF THE INVENTION

In modern biotechnology, it is common practice to clone DNA sequences from biological organisms of any type and then to introduce genetic constructs carrying those sequences into plasmids or viral vectors for replication *in vitro*. Often these sequences are assembled into expression vectors which are then introduced into and expressed in foreign hosts of any of a number of organisms both eukaryotic and prokaryotic. In its isolated form, DNA can be studied, and its sequence can be determined. From a DNA sequence the structure and encoding capacity and other attributes of the DNA can be analyzed. It is also possible to synthesize altered and/or synthetic DNA sequences to make new gene products and to alter the genetic sequence of organisms both large and small.

In the process of expressing a coding sequence of DNA to make a protein, a first step involves the process of transcription whereby a messenger RNA sequence is made, which is ultimately translated into protein. Since the DNA and RNA are essential parts of the protein production process, it is undesirable during the process of *in vitro* cloning and expression of these nucleotides that the

nucleotide chains be degraded. Nevertheless, since imperfectly purified biological reagents are conventionally used in such *in vitro* processes, the inadvertent introduction of unwanted enzymes is a very practical problem. All organisms make in their cells enzymes known as ribonucleases, which have the principal function of degrading nucleic acids in the cells. Such degradation is an essential part of biological processes both to down regulate messenger RNA which is no longer desired and also as a part of the cellular recycling process in which the component parts of nucleic acids are reused to synthesize other nucleic acids. Therefore, ribonucleases are ubiquitous in biological organisms. Ribonucleases also tend to be stable and highly active. Even trace amounts of ribonucleases can be lethal to *in vitro* DNA expression systems or systems for handling, utilizing or characterizing RNA, since even a trace amount of a ribonuclease can rapidly degrade all of the mRNA in an experimental sample.

Accordingly, companies which specialize in selling products to researchers in modern biotechnology supply reagents which are specifically intended to help overcome the problem of contaminating trace ribonucleases. Several companies sell, for example, ribonuclease-free water. There is a market for ribonuclease-free water because normal tap water can often be contaminated with extremely small amounts of ribonucleases, which can nevertheless severely disrupt experiments sensitive to such enzymatic activity. Experimenters hands contain ribonucleases which, through insufficiently careful lab techniques, can be introduced to and contaminate the results of carefully done *in vitro* experiments. Thus the avoidance of contamination by the action of ribonucleases is a significant consideration in many types of experiments in molecular biology.

Ribonucleases can be inhibited by protein molecules produced by cells, the specific purpose of which is to

inhibit the enzymatic activity of a ribonuclease. Such proteins are called, naturally enough, ribonuclease inhibitors (or RI). The desirability of ribonuclease inhibitors for use in laboratory techniques of modern biotechnology has led to purified ribonuclease inhibitors being commercial products currently sold on the market by several reagent supply companies. Ribonuclease inhibitor can be isolated from many types of cells, notably most conveniently from placental cells, or it can be created by *in vitro* expression of DNA sequence which encodes ribonuclease inhibitor. US Patent No. 5,552,302 describes methods for the production of human recombinant placental ribonuclease inhibitor in prokaryotic cells.

It is a limitation on the ribonuclease inhibitors currently on the market place that they are not very stable, and certainly not as stable as the ribonucleases which they inhibit. Ribonuclease inhibitors tend to be susceptible to rapid oxidation. The oxidation of the ribonuclease inhibitor is a rapid cascading process which is irreversible. The ribonuclease inhibitor has to be completely reduced to bind to a ribonuclease. Since oxygen is, of course, prevalent in the environment, as are many oxidizing agents, this oxidation sensitivity is a severe limitation on the use of ribonuclease inhibitors that reduces their convenient use in laboratory practice of modern techniques of biotechnology. Accordingly, a ribonuclease inhibitor having less susceptibility to oxidation would be more advantageous, because it would be more stable and therefore more likely to decrease the loss of valuable nucleotides to the activity of unwanted ribonucleases.

BRIEF SUMMARY OF THE INVENTION

The present invention is summarized in a mutant ribonuclease inhibitor which has been modified so as to change cysteine residues from the native form of the ribonuclease inhibitor to other amino acids that will not

form disulfide bonds. These changes to the amino acid sequence are directed to the location of adjacent cysteine residues in the sequence of ribonuclease inhibitor. Adjacent cysteine residues naturally occur in many, although not all, ribonuclease inhibitors.

It is an object of the present invention to provide a mutant form of a ribonuclease inhibitor that is less susceptible to oxidation and therefore more stable in its use in inhibiting the activity of ribonucleases in molecular biology procedures.

It is a feature of the present invention in that modifications of the sequence of ribonuclease inhibitors which include modifying cysteine residues where they are adjacent to each other results in mutant forms of ribonuclease inhibitor that still have appropriate specificity and binding affinity to ribonucleases but are more resistant to oxidation.

Other objects, advantages, and features of the present invention will become apparent from the following specification when taken in conjunction with the accompanying drawings.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 is a graphical representation of the three dimensional structure of human ribonuclease inhibitor.

Fig. 2 illustrates the change in a peptide bond from a trans to a cis configuration.

Fig. 3 is a graphical illustration of some of the results of tests conducted as described in the examples below.

Fig. 4 is a graphical illustration of additional experimental results.

Fig. 5 is a graphical illustration of yet additional experimental results.

Fig. 6 is another graphical illustration of results from the examples below.

Fig. 7 illustrates the sequence comparisons among

several ribonuclease inhibitors.

DETAILED DESCRIPTION OF THE INVENTION

The work described herein is based on a premise. The premise is that the observed instability of ribonuclease molecules occurs because of oxidation of cysteine residues to form disulfide bridges. The thesis is further that such disulfide bridges are most likely to form when cysteine residues containing unpaired thiol groups are closely adjacent to each other. Therefore, in accordance with the method described herein, the amino acid sequence of a ribonuclease inhibitor molecule is re-designed so as to avoid having cysteine residues which are adjacent or closely adjacent in the engineered ribonuclease inhibitor. It has been found that by making this change to the amino acid sequence of ribonuclease inhibitor, and thus forming mutant ribonuclease inhibitors, those mutant ribonuclease inhibitors are more oxidation resistant and have a greater stability during handling than the wild-type ribonuclease inhibitor on which they are based. In this way, the use of ribonuclease inhibitors in biological processes becomes more practical, as the ribonuclease inhibitors become more stable and require less special treatment in order to remain active.

It is a notable feature of ribonuclease inhibitor molecules that they are typically rich in cysteine residues. The human ribonuclease inhibitor is a 50 kilodalton molecule composed of 460 amino acids, of which 32 are cysteine residues. All of the cysteine residues must remain reduced for the human ribonuclease inhibitor to bind to a ribonuclease.

An illustration of the three dimensional structure of the human ribonuclease inhibitor is illustrated in Fig. 1. The sequence of the ribonuclease inhibitor can be found in Lee et al. Biochemistry 27:8545-8553 (1988), the disclosure of which is hereby incorporated by reference. From both Figure 1 and the sequence of the protein, it can be readily

seen that some of the cysteine residues are located adjacent to each other. The amino acid residues at positions numbered 95 and 96 and 328 and 329 in the human RI sequence are all cysteines. It was theorized that these cysteine residues would be the most likely to be oxidized to form disulfide bonds which would interfere with the biological activity of the molecule.

When adjacent cysteine residues form a disulfide bond between them, the two cysteine residues together form an eight member ring that includes a polypeptide bond. Normally in a protein, a trans peptide bond is energetically favored over a cis peptide bond. A trans bond is, however, especially unstable in an eight-membered ring. It was demonstrated some time ago that trans-cyclooctene is much less stable than cis-cyclooctene, Turner and Meador J. Am. Chem. Soc. 79:4133-4136 (1957). This conformational energy analysis suggests that a peptide bond within a disulfide bond formed between adjacent cysteine residues will exist in the cis rather than the trans conformation. This is illustrated in Figure 2. The strain in the cis peptide bond is overcome by the strength of the covalent disulfide bond. Indeed, oxidized cysteine residues with such cis peptide bonds have been found in crystalline methanol dehydrogenase and crystalline peptides. Blake, Nature Struct. Biol. 1:101-105; 557 (1994); Mez, Crystl. Struct. Comm., 3:657-660 (1993). In solution, the peptide bond between two adjacent oxidized cysteine residues appear to be in conformational equilibrium, with either the trans conformation or the cis conformation predominating.

The formation of a disulfide bond between adjacent cysteine residues has structural consequences for the protein molecule in which they reside. In particular, a cis peptide bond is not tolerated in either an α- helix or β- sheet three-dimensional structure. Replacing a trans peptide bond with a cis peptide bond is therefore likely to distort the tertiary structure of a native protein. In

human ribonuclease inhibitor, this distortion could bring together other pairs of cysteine residues, leading to further oxidation, and a cascade of transformational shape change. It was for this reason that it was decided to modify the coding sequence for the human ribonuclease inhibitor to remove adjacent cysteine residues, to prevent the formation of unwanted disulfide bonds between adjacent cysteine residues.

In native human ribonuclease inhibitor, the two pairs of cysteine residues which lie most adjacent to each other are the cysteines at amino acids 94 and 95 (which are in a loop) and the cysteines at amino acid 328 and 239 which reside in an α-helix. None of these four cysteine residues are in contact with angiogenin in the complex which forms between human ribonuclease inhibitor and angiogenin. Porcine ribonuclease inhibitor has one pair of adjacent cysteine residues, which are homologous to the cysteines at residues 328 and 329 in the human RI sequence. In contrast, ribonuclease inhibitor from rat has no pairs of adjacent cysteine residues. The oxidative stability of the rat ribonuclease inhibitor protein, as well as its three-dimensional structure, is currently not known.

As will be discussed with the experimental results below, it was found possible to inhibit the formation of disulfide bonds between adjacent cysteine residues of a ribonuclease inhibitor by replacing the adjacent cysteine residues with alanine residues. The mutant human pancreatic ribonuclease inhibitor molecules thus created, have pairs of alanine-for-cysteine substitutions at both amino acids 94 and 95, at both amino acid positions 238 and 239, or substitutions for all four of the cysteine residues. It was demonstrated that the replacing of any or all of the cysteine residues with alanine did not markedly impair the ability of the human ribonuclease inhibitor to bind RNase A. There was, however, some slight diminution in affinity to ribonuclease for some of the variants.

It was discovered, however, that replacing these

adjacent cysteine residues with alanine residues made the human ribonuclease inhibitor significantly more oxidation resistant as compared to the wild-type protein. Oxidation resistance was tested using hydrogen peroxide based on ease of laboratory use. It was discovered that the wild-type human ribonuclease inhibitor loses 50% of its activity in a solution which has little as 0.007% hydrogen peroxide volume-per-volume. By contrast, the mutant ribonuclease inhibitor having alanine substitutions at amino acid positions 328 and 329 retain 50% of its ribonuclease inhibitor activity at 0.09% volume-per-volume hydrogen peroxide. By this measure, the mutant C328/C329A ribonuclease inhibitor variant is 10 to 15 fold more resistant to oxidative damage than is the wild-type human ribonuclease inhibitor.

In this way, it is possible to create mutants of wild-type human ribonuclease inhibitor which are more oxidation resistant than the wild types. Such oxidation resistance is created by the substitution of another amino acid for at least one of the adjacent cysteine residues within the molecule. Such oxidation resistant variants of ribonuclease inhibitor are useful for a wide variety of laboratory protocols which now would avoid the need for reducing agents in reactions containing ribonuclease inhibitor. The mutant variants of human ribonuclease inhibitor could serve another purpose. In addition to binding to ribonucleases, the ribonuclease inhibitor also binds to angiogenin. Angiogenin promotes neovascularization which is the formation of new blood vessels, and human ribonuclease inhibitor has been shown effective in inhibiting angiogenin mediated vascularization. In such physiological experiments, human ribonuclease inhibitor is exposed to an oxidative environment, and it is known that such an environment can compromise its ability to inhibit angeniogenin. It is therefore likely that the oxidation resistant variants of ribonuclease inhibitor as described herein would be more

effective than wild-type human ribonuclease inhibitor at inhibiting angiogenin mediated angiogenesis. That prospect has both clinical and diagnostic implications.

The methodology disclosed here will be equally effective for ribonuclease inhibitor molecules from other species. Shown in Fig. 7 is a comparison of the amino acid sequences of RNASE inhibitor from rat, pig, and human. Note that pig RI shares the adjacent cysteine residues (at positions 323 and 324) corresponding to residues 328 and 329 of the human sequence, and thus could be modified as described here. The technique described here will work with all such RI molecules that natively have adjacent cysteine residues. Some RI variants, like the rat molecule illustrated in Fig. 7, contain no adjacent cysteines.

Examples

The goal of the work described below was to create mutant forms of human ribonuclease inhibitor which would hinder the cataclysmic oxidation of human ribonuclease inhibitor. Reasoning that the formation of disulfide bonds amongst cysteine residues in the human ribonuclease inhibitor molecule would be most likely to occur among those residues which were closest in space, it was decided to survey the three-dimensional structure of ribonuclease inhibitor to determine those residues which were closest to each other in the normal three-dimensional conformational structure of human ribonuclease inhibitor. Figure 1 is an illustration of the 3D model that was used for the structure of human ribonuclease inhibitor. Study of that structure revealed that the most proximal cysteine residues in native human ribonuclease inhibitor are those which are adjacent in the primary amino acid sequence as published by Lee et al. (*Biochemistry* 27:8545-8553 (1988)). The close amino acid residues were the cysteines at amino acid positions 94 and 95, which are in a loop structure, and the cysteines at residues 328 and 329, which are part of an alpha helix structure. None of these four cysteine

residues contacts angiogenin during the formation of the ribonuclease inhibitor complex with angiogenin. It was observed that porcine ribonuclease inhibitor varies from the human sequence in that it has only one pair of adjacent cysteine residues, which are homologous to cysteines 328 and 329 in the human ribonuclease inhibitor complex. By contrast, rat ribonuclease inhibitor has no pairs of adjacent cysteine residues, but the oxidative stability of the rat protein, as well as its three-dimensional structure, is currently unknown.

When adjacent cysteine residues form a disulfide bond, the resulting cysteine residues define an eight-membered ring that includes a peptide bond. Normally in a protein a trans (i.e. *Z*) peptide bond is more favored energetically as compared to a cis (i.e. *E*) peptide bond. A trans bond is, however, especially unstable in an eight-membered ring. These conformational energetics suggest that a peptide bond within a disulfide bond formed between adjacent cysteine residues would tend to be in the cis, rather than the trans, conformation. This is illustrated in Figure 2.

The strain of the cis peptide bond is overcome by the strength of the covalent disulfide bond which has a disassociation energy equal to 65 kcal/mol in $H_3CS\text{-}SCH_3$. Indeed such cysteine residues with cis peptide bonds have been found in crystalline methanol dehydrogenase and crystalline peptides. Further, the stability of an intramolecular disulfide bond in the generalized structure Cys-$(Ala)_n$-Cys peptides is greater for n=0 than for n=2, 4, or 5 (Zhang and Schneider, *J. Bio. Chem.* 264:18472-18479 (1989)). In solution, the peptide bond within adjacent disulfide-bonded cysteine residues appears to be in conformational equilibrium with either the trans conformation or the cis confirmation predominating.

The formation of a disulfide bond between adjacent cysteine residues has structural implications for the overall protein molecule of which it is a part. In particular, a cis peptide bond is inconsistent with an α-

helix or a β-sheet secondary structure. Replacing a trans peptide bond with a cis peptide bond distorts the structure of the native protein. In human ribonuclease inhibitor, distortion could congregate other pairs of cysteine residues leading to further oxidation and potentially catastrophic degradation of the biologically active form in the molecule.

RNASE A for use in this work was produced in *Escherichia coli* with a recombinant DNA expression system, as described in delCardayre et al., Protein Engng. 8:261-273 (1995). Wild-type hRI and its variants were produced in *E. coli* by using plasmid pET-RI, which directs the expression of hRI as described in Leland et al., Proc. Natl. Acad. Sci. USA 95:10407-10412 (1998). To produce hRI variants, the cDNA that codes for hRI was mutated by the method of Kunkel et al. Methods Enzymol. 154:367-382 (1987). The oligonucleotides used were BMK14 (C94A/C95A; *Hind*III):GGCCCCCGTCAG**CGCCGC**GTTCTGGAGGCT**AAGCTT**CTG; BMK16 (C328A/C329A; *Nhe*I):GCTGAAGTGG**CTAGCGGC**GGCGGCTGTGAA; BMK17(C328A; *Sph*I):GCTGAAGTGGGA**GCATGC**GGCGGCTGTGAA; and BMK18(C329A; *Nhe*I):GCTGAAGTGG**CTAGC**GCAGGCGGCTGTGAA. In these sequences, the reverse complement of new alanine codons is in bold type and new restriction endonuclease sites are underlined. cDNA sequences of mutated plasmids were determined with an ABI 373 Automated Sequencer.

Wild-type hRI and the variants were produced and purified essentially as described (Leland et al., supra). The key step in the purification protocol is affinity chromatography on RNASE A-Sepharose 4B resin. Briefly, *E. coli* lysate in 50 mM potassium phosphate buffer, pH 7.5, containing glycerol (15% v/v), DTT (5 mM), and EDTA (1 mM) was loaded onto the resin. Only active molecules of hRI are bound by the immobilized RNASE A. The loaded resin was washed with 50 mM potassium phosphate buffer, pH 7.5, containing NaCl (0.5 M) and DTT (8 mM), and eluted with 0.10 M sodium acetate buffer, pH 5.0, containing glycerol (15% v/v), NaCl (3.0 M), and DTT (8 mM).

The presence of 8 mM DTT would interfere in assays of oxidation resistance. To prepare hRI for the assays described below, the concentration of DTT was reduced by 10^3-fold by concentration/dilution. Briefly, hRI was concentrated 10-fold by ultrafiltration using a Microcon 10 micron concentrator from Amicon (Beverly, MA). The resulting solution was diluted 10-fold with degassed 20 mM HEPES-HCl buffer, pH 7.6, containing glycerol (50% v/v) and KCl (50 mM). This treatment was repeated three times. hRI thus treated retains full activity, provided that its exposure to air is minimal.

Concentrations of RNASE A were determined by assuming that A=0.72 at 277.5 nm for a 1.00 mg/mL solution. Concentrations of hRI were determined by assuming that A=0.88 at 280 nm for a 1.00 mg/mL solution as described in Ferreras et al., J. Biol. Chem. 270:28570-28578 (1995). Concentrations of poly(cytidylic acid)[poly(C)] were determined by assuming that ϵ=6200 $M^{-1}cm^{-1}$ per nucleotide at 268 nm as per Yakovlev et al., Eur. J. Biochem. 204:187-190 (1992).

To assay for inhibition of RNASE A, serial dilutions were made to produce six solutions (10μL each) of 20 mM HEPES-HCl buffer, pH 7.6, containing KCl (50 MM) and hRI (10 nM-10μM). A solution (10μl) of 20 mM HEPES-HCl buffer, pH 7.6, containing KCl (50mM) and RNASE A (80 nM) was added to each of the hRI solutions. The resulting mixtures were incubated at 37°C for 5 min. The ribonucleolytic activity in each mixture was then assessed by using a spectrophotometric assay for poly(C) cleavage, as described (delCardayre et al., 1995), with [poly(C)]=37 μM. This experiment was performed at least twice with wild-type hRI and each variant.

To test for oxidation resistance, serial dilutions were made to produce seven solutions (5μL each) of 20 mM HEPES-HCl buffer, pH 7.6, containing KCl (50 mM) and H_2O_2 (0.004-2% v/v, which is 2 mM - 0.9M). A solution (5μl) of 20 mM HEPES-HCl buffer, pH 7.6, containing KCl (50 mM) and

hRI (10 μM) was added to each of the H_2O_2 solutions. The resulting mixtures were incubated at 37 °C for 30 min. A solution (10 μL) of 20 mM HEPES-HCl buffer, pH 7.6, containing KCl (50 mM) and RNASE A (80 nM) was then added to each H_2O_2 plus hRI solution. The resulting mixtures were incubated at 37°C for 5 min. The ribonucleolytic activity in each mixture was then assessed by using a spectrophotometric assay for poly(C) cleavage as described (delCardayre et la., 1995), with [poly(C)]=37 μM. This experiment was performed at least twice with wild-type hRI and each variant.

It has been previously reported that the cysteines at residues 94, 95, 328, and 329 of hRI do not contact angiogenin in the complex formed between human ribonuclease inhibitor and angiogenin. Thus it was not anticipated that replacing any of these cysteine residues with alanine would significantly impair the ability of human ribonuclease inhibitor to bind to ribonuclease A. Shown in Figures 3 and 4 is a graphical representation of the data showing the ability of the various modified or mutant human ribonuclease inhibitors to inhibit ribonucleolytic activity. These results demonstrate that none of the substitutions significantly impair the ability of the mutant forms of human ribonuclease inhibitor to bind to ribonuclease A. However, C94A/C95A hRI (human ribonuclease inhibitor with cysteines at 94 and 95 substitute by alanine) and C94A/C95A/C328A/C329A hRI are slightly less effective inhibitors of ribonuclease activity than is the variant C328A/C329A. The affinity of the two single amino acid mutations variants, C328A hRI and C329A hRI for human ribonuclease inhibitor is between that of the wild-type human ribonuclease inhibitor and the C328A/C329A variant as shown in Figure 4.

The test for oxidation resistance demonstrated that replacing adjacent cysteine residues with alanine makes the resulting mutant hRI oxidation resistant. As the oxidant in this test we chose H_2O_2, which is easier to dispense than

O_2 gas and which likewise oxidizes thiols to disulfides. As shown in Figure 5, H_2O_2 has a greater effect on C328A/C329A human ribonuclease inhibitor than it has on the C94A/C95A variant. In our assays, wild-type human ribonuclease inhibitor loses 50% of its activity at 0.007% volume per volume H_2O_2. By contrast, C328A/C329A mutant human ribonuclease inhibitor retains 50% of its activity at 0.09% volume per volume H_2O_2. By this measure, the C328A/C329A mutant form of ribonuclease inhibitor is ten to fifteen times more resistant to oxidative damage than is the wild-type human ribonuclease inhibitor.

The enhanced oxidation resistance of C328A/C329A mutant hRI appears to result from the inhibition of the formation of a disulfide bond between the cysteines which would otherwise reside at residues 328 and 329. As shown in Figure 5, the individual C328A and C329A variants of mutant variants of hRI are just as resistant to oxidation by H_2O_2 as is the C328A/C329A form of hRI. The simplest explanation for this result is that oxidation of the wild-type protein results in a cys-328-Cys 329 disulfide bond which cannot form in either of the single amino acid variants C328A or C329A, or in the double amino acid variant C328A/C329A.

High levels of H_2O_2 (such as the 0.09% volume per volume, which equals 0.04M) inactivate all five forms of mutant human ribonuclease inhibitor. At least two explanations are possible for this result. Disulfide bonds can form between thiols of nonadjacent cysteine residues. Alternatively, thiols of hRI that contact RNASE A in the hRI complex with RNASE A could be oxidized to form sulfonates (RSO_3^-). Such over-oxidation is more likely with H_2O_2 than with diatomic oxygen gas.

Currently commercial human ribonuclease inhibitor is distributed in solutions containing millimolar levels of dithiothreitol (DTT). The presence of this reducing agent is included with the ribonuclease inhibitor to maintain the hRI in a reduced, and hence active, form. In many

instances, such reducing agents are incompatible with laboratory protocols. Moreover, reducing agents are oxidized and thus rendered ineffective by the ubiquitous oxidant oxygen gas and transition metal ions. We find that replacing only one (i.e. Cys328 or Cys 329) of the 32 cysteine residues in hRI with an alanine residue substantially increases the resistance of the molecule to oxidation, without compromising its affinity for RNAase A. This demonstrates that variants of hRI lacking a cysteine residue at positions 328 or 329, or the homologous positions in other ribonuclease inhibitors, will be more useful than wild-type ribonuclease inhibitors in many laboratory protocols.

Oxidation resistant variants of ribonuclease inhibitor can serve another purpose. Angiogenin, like ribonuclease A, is tightly bound by ribonuclease inhibitor. As its name implies, angiogenin promotes neovascularization or the formation of new blood vessels. Ribonuclease inhibitor has been shown to be effective in inhibiting angiogenin-mediated neovascularization. In psychological experiments ribonuclease inhibitor is exposed to an oxidative environment, which could compromise its ability to inhibit angiogenin. This phenomenon would indicate that oxidation resistant variants, such as those described herein, would be more effective than wild-type ribonuclease inhibitor at inhibiting angiogenin-mediated angiogenesis.

CLAIM OR CLAIMS

I/WE CLAIM:

1. A mutant ribonuclease inhibitor having at least one amino acid substitution in at least one of its adjacent cysteine residues to an amino acid residue not capable of forming a disulfide bond, the mutant ribonuclease inhibitor having a greater resistance to oxidation, the mutant ribonuclease inhibitor retaining its specificity and binding affinity to ribonuclease.

2. The ribonuclease inhibitor of claim 1, wherein ribonuclease inhibitor is a human ribonuclease inhibitor and the substituted cysteine residue is in at least one of positions 94, 95, 328 and 329.

3. The ribonuclease inhibitor of claim 1, wherein the cysteine residue is replaced with an alanine residue.

4. The ribonuclease inhibitor of claim 1, wherein the substitution in at least one of the cysteine residues inhibits the formation of a disulfide bond with an adjacent cysteine residue.

5. The ribonuclease inhibitor of claim 1, wherein the mutant ribonuclease inhibitor is 10 to 15 fold more resistant to oxidative damage than the native human ribonuclease inhibitor.

6. The ribonuclease inhibitor of claim 1, wherein the ribonuclease is of the RNASE A superfamily.

7. The ribonuclease inhibitor of claim 1, wherein the modified ribonuclease inhibitor exhibits an *in vitro* inhibition of ribonucleolytic activity.

8. The ribonuclease inhibitor of claim 1, wherein the mutant ribonuclease inhibitor is derived from the native human ribonuclease inhibitor.

9. A mutant human ribonuclease inhibitor having at least one amino acid substitution in at least one of its adjacent cysteine residues, the substitution being an amino acid other than cysteine, the mutant ribonuclease inhibitor having a greater resistance to oxidation, the mutant ribonuclease inhibitor retaining the specificity and binding affinity to angiogenin of the wild-type human ribonuclease inhibitor.

10. The ribonuclease inhibitor of claim 9, wherein the substituted cysteine residue is in at least one of positions 94, 95, 328 and 329.

11. A DNA sequence comprising a coding sequence encoding a mutant ribonuclease inhibitor which differs from the corresponding wild-type ribonuclease inhibitor in that at least one codon for cysteine has been replaced by a codon for another amino acid.

12. A DNA sequence as claimed in claim 11 wherein the replaced cysteine residue is adjacent to another cysteine residue in the wild-type sequence.

13. A DNA sequence as claimed in claim 11 wherein the ribonuclease inhibitor is human ribonuclease inhibitor and the cysteine replaced is at least one of amino acid positions 94, 95, 328 and 329.

14. A DNA sequence as claimed in claim 11 wherein the substitution is a codon for alanine.

ABSTRACT OF THE DISCLOSURE

Mutant forms of ribonuclease inhibitor are described which are rendered more resistant to oxidation while retaining affinity for both ribonuclease and angiogenin. The mutant forms have another amino acid, typically an alanine, substituted for one or more of the adjacent cysteine residues in the wild-type sequence to prevent the formation of unwanted disulfide bonds which can disrupt the effectiveness of the molecule.

SEQUENCE LISTING

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             20                  25                  30

Cys Gly Leu Thr Glu Val Arg Cys Lys Asp Ile Arg Ser Ala Ile Gln
         35                  40                  45

Ala Asn Pro Ala Leu Thr Glu Leu Ser Leu Arg Thr Asn Glu Leu Gly
     50                  55                  60

Asp Ala Gly Val Gly Leu Val Leu Gln Gly Leu Gln Asn Pro Thr Cys
 65                  70                  75                  80

Lys Ile Gln Lys Leu Ser Leu Gln Asn Cys Ser Leu Thr Glu Ala Gly
                 85                  90                  95

Cys Gly Val Leu Pro Asp Val Leu Arg Ser Leu Ser Thr Leu Arg Glu
            100                 105                 110

Leu His Leu Asn Asp Asn Pro Leu Gly Asp Glu Gly Leu Lys Leu Leu
        115                 120                 125

Cys Glu Gly Leu Arg Asp Pro Gln Cys Arg Leu Glu Lys Leu Gln Leu
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```
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Phe His Glu Ala Gly Ile His Thr Leu Cys Gln Gly Leu Lys Asp Ser
            180                 185                 190

Ala Cys Gln Leu Glu Ser Leu Lys Leu Glu Asn Cys Gly Ile Thr Ser
        195                 200                 205

Ala Asn Cys Lys Asp Leu Cys Asp Val Val Ala Ser Lys Ala Ser Leu
    210                 215                 220

Gln Glu Leu Asp Leu Gly Ser Asn Lys Leu Gly Asn Thr Gly Ile Ala
225                 230                 235                 240

Ala Leu Cys Ser Gly Leu Leu Leu Pro Ser Cys Arg Leu Arg Thr Leu
                245                 250                 255

Trp Leu Trp Asp Cys Asp Val Thr Ala Glu Gly Cys Lys Asp Leu Cys
            260                 265                 270

Arg Val Leu Arg Ala Lys Gln Ser Leu Lys Glu Leu Ser Leu Ala Gly
        275                 280                 285

Asn Glu Leu Lys Asp Glu Gly Ala Gln Leu Leu Cys Glu Ser Leu Leu
    290                 295                 300

Glu Pro Gly Cys Gln Leu Glu Ser Leu Trp Val Lys Thr Cys Ser Leu
305                 310                 315                 320

Thr Ala Ala Ser Cys Pro His Phe Cys Ser Val Leu Thr Lys Asn Ser
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Ser Leu Phe Glu Leu Gln Met Ser Ser Asn Pro Leu Gly Asp Ser Gly
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Val Val Glu Leu Cys Lys Ala Leu Gly Tyr Pro Asp Thr Val Leu Arg
        355                 360                 365

Val Leu Trp Leu Gly Asp Cys Asp Val Thr Asp Ser Gly Cys Ser Ser
    370                 375                 380

Leu Ala Thr Val Leu Leu Ala Asn Arg Ser Leu Arg Glu Leu Asp Leu
385                 390                 395                 400
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            20                  25                  30

Cys Gly Leu Thr Glu Glu His Cys Lys Asp Ile Gly Ser Ala Leu Arg
        35                  40                  45

Ala Asn Pro Ser Leu Thr Glu Leu Cys Leu Arg Thr Asn Glu Leu Gly
    50                  55                  60

Asp Ala Gly Val His Leu Val Leu Gln Gly Leu Gln Ser Pro Thr Cys
65                  70                  75                  80

Lys Ile Gln Lys Leu Ser Leu Gln Asn Cys Ser Leu Thr Glu Ala Gly
                85                  90                  95

Cys Gly Val Leu Pro Ser Thr Leu Arg Ser Leu Pro Thr Leu Arg Glu
            100                 105                 110

Leu His Leu Ser Asp Asn Pro Leu Gly Asp Ala Gly Leu Arg Leu Leu
        115                 120                 125

Cys Glu Gly Leu Leu Asp Pro Gln Cys His Leu Glu Lys Leu Gln Leu
    130                 135                 140

Glu Tyr Cys Arg Leu Thr Ala Ala Ser Cys Glu Pro Leu Ala Ser Val
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Ile Gly Glu Ala Gly Ala Arg Val Leu Gly Gln Gly Leu Ala Asp Ser
            180                 185                 190

Ala Cys Gln Leu Glu Thr Leu Arg Leu Glu Asn Cys Gly Leu Thr Pro
        195                 200                 205

Ala Asn Cys Lys Asp Leu Cys Gly Ile Val Ala Ser Gln Ala Ser Leu
    210                 215                 220

Arg Glu Leu Asp Leu Gly Ser Asn Gly Leu Gly Asp Ala Gly Ile Ala
225                 230                 235                 240

Glu Leu Cys Pro Gly Leu Leu Ser Pro Ala Ser Arg Leu Lys Thr Leu
                245                 250                 255

Trp Leu Trp Glu Cys Asp Ile Thr Ala Ser Gly Cys Arg Asp Leu Cys
            260                 265                 270

Arg Val Leu Gln Ala Lys Glu Thr Leu Lys Glu Leu Ser Leu Ala Gly
        275                 280                 285

Asn Lys Leu Gly Asp Glu Gly Ala Arg Leu Leu Cys Glu Ser Leu Leu
    290                 295                 300

Gln Pro Gly Cys Gln Leu Glu Ser Leu Trp Val Lys Ser Cys Ser Leu
305                 310                 315                 320

Thr Ala Ala Cys Cys Gln His Val Ser Leu Met Leu Thr Gln Asn Lys
                325                 330                 335

His Leu Leu Glu Leu Gln Leu Ser Ser Asn Lys Leu Gly Asp Ser Gly
            340                 345                 350

Ile Gln Glu Leu Cys Gln Ala Leu Ser Gln Pro Gly Thr Thr Leu Arg
        355                 360                 365

Val Leu Cys Leu Gly Asp Cys Glu Val Thr Asn Ser Gly Cys Ser Ser
    370                 375                 380

Leu Ala Ser Leu Leu Leu Ala Asn Arg Ser Leu Arg Glu Leu Asp Leu
385                 390                 395                 400

Ser Asn Asn Cys Val Gly Asp Pro Gly Val Leu Gln Leu Leu Gly Ser
                405                 410                 415
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Ser Asn Glu Leu Gly Asp Val Gly Val His Cys Val Leu Gln Gly Leu
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Gln Thr Pro Ser Cys Lys Ile Gln Lys Leu Ser Leu Gln Asn Cys Cys
85 90 95

Leu Thr Gly Ala Gly Cys Gly Val Leu Ser Ser Thr Leu Arg Thr Leu
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Pro Thr Leu Gln Glu Leu His Leu Ser Asp Asn Leu Leu Gly Asp Ala
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Gly Leu Gln Leu Leu Cys Glu Gly Leu Leu Asp Pro Gln Cys Arg Leu
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Glu Lys Leu Gln Leu Glu Tyr Cys Ser Leu Ser Ala Ala Ser Cys Glu
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Pro Leu Ala Ser Val Leu Arg Ala Lys Pro Asp Phe Lys Glu Leu Thr

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                  165                 170                 175

Val Ser Asn Asn Asp Ile Asn Glu Ala Gly Val Arg Val Leu Cys Gln
             180                 185                 190

Gly Leu Lys Asp Ser Pro Cys Gln Leu Glu Ala Leu Lys Leu Glu Ser
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Cys Gly Val Thr Ser Asp Asn Cys Arg Asp Leu Cys Gly Ile Val Ala
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Ser Lys Ala Ser Leu Arg Glu Leu Ala Leu Gly Ser Asn Lys Leu Gly
225                 230                 235                 240

Asp Val Gly Met Ala Glu Leu Cys Pro Gly Leu Leu His Pro Ser Ser
                 245                 250                 255

Arg Leu Arg Thr Leu Trp Ile Trp Glu Cys Gly Ile Thr Ala Lys Gly
             260                 265                 270

Cys Gly Asp Leu Cys Arg Val Leu Arg Ala Lys Glu Ser Leu Lys Glu
         275                 280                 285

Leu Ser Leu Ala Gly Asn Glu Leu Gly Asp Glu Gly Ala Arg Leu Leu
     290                 295                 300

Cys Glu Thr Leu Leu Glu Pro Gly Cys Gln Leu Glu Ser Leu Trp Val
305                 310                 315                 320

Lys Ser Cys Ser Phe Thr Ala Ala Cys Cys Ser His Phe Ser Ser Val
                 325                 330                 335

Leu Ala Gln Asn Arg Phe Leu Leu Glu Leu Gln Ile Ser Asn Asn Arg
             340                 345                 350

Leu Glu Asp Ala Gly Val Arg Glu Leu Cys Gln Gly Leu Gly Gln Pro
         355                 360                 365

Gly Ser Val Leu Arg Val Leu Trp Leu Ala Asp Cys Asp Val Ser Asp
     370                 375                 380

Ser Ser Cys Ser Ser Leu Ala Ala Thr Leu Leu Ala Asn His Ser Leu
385                 390                 395                 400

Arg Glu Leu Asp Leu Ser Asn Asn Cys Leu Gly Asp Ala Gly Ile Leu
                 405                 410                 415

Gln Leu Val Glu Ser Val Arg Gln Pro Gly Cys Leu Leu Glu Gln Leu
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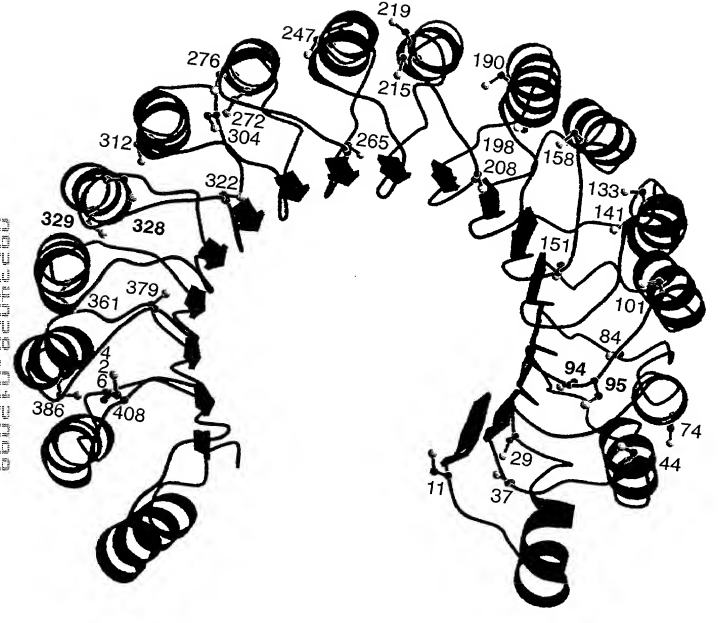
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FIG 1

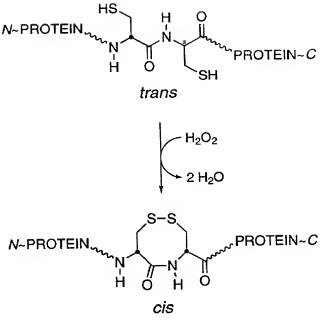
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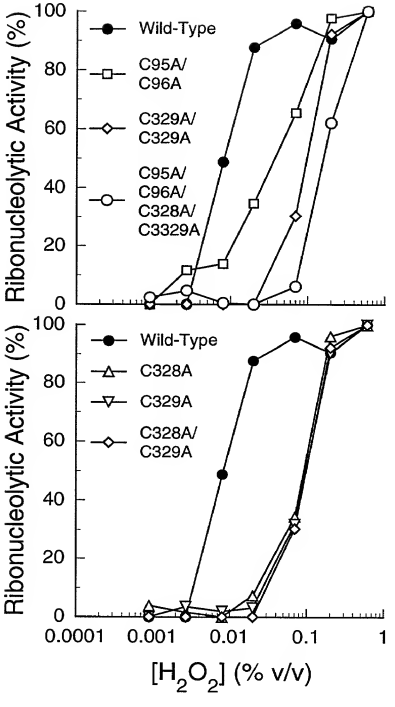
FIG 2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C96A/
C328A/
C3329A
Ribonucleolytic Activity (%)
100
80
60
40
20
0
Wild-Type
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FIG 3

FIG 4

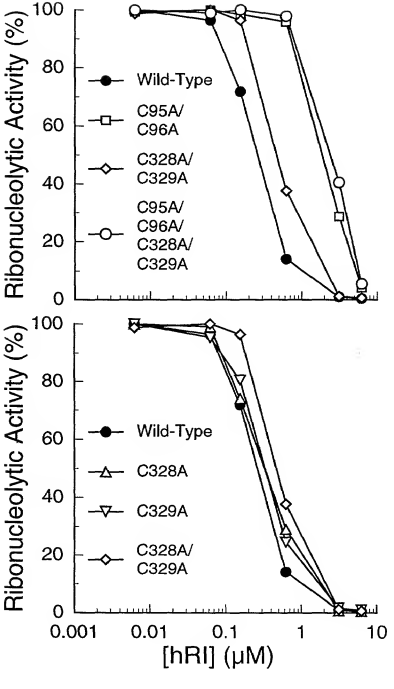

FIG 5

FIG 6

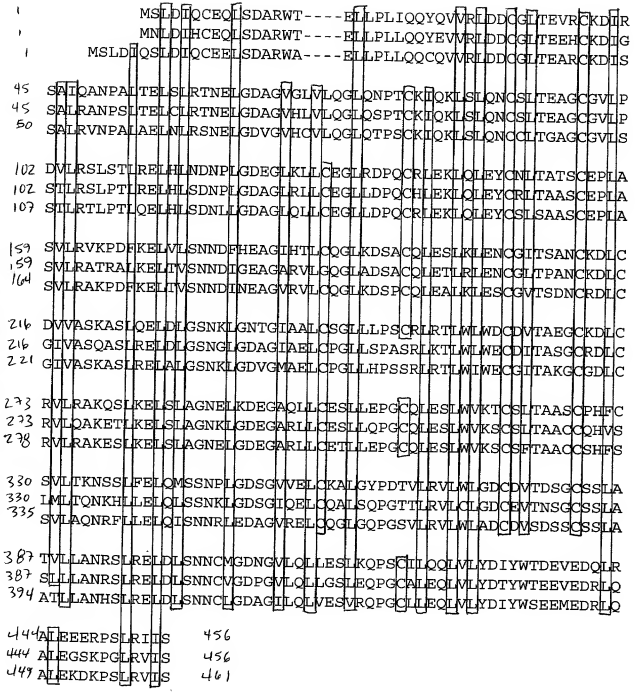

FIG 7

QBMAD\173574

PTO/SB/01 (6-95)
Approved for use through 9/30/98. OMB 0651-0032
Patent and Trademark Office: U.S. DEPARTMENT OF COMMERCE

Please type a plus sign (+) inside this box [+]

0010/PTO
Rev. 6/95

U.S. Department of Commerce
Patent and Trademark Office

DECLARATION FOR UTILITY OR DESIGN PATENT APPLICATION

[X] Declaration Submitted with Initial Filing OR [ ] Declaration Submitted after Initial Filing

Attorney Docket Number	960296.95360
First Named Inventor	Ronald T. Raines
COMPLETE IF KNOWN	
Application Number	
Filing Date	
Group Art Unit	
Examiner Name	

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name.

I believe that I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled:

OXIDATION-RESISTANT RIBONUCLEASE INHIBITOR

(Title of the invention)

the specification of which

[X] is attached hereto

OR

[ ] was filed on (MM/DD/YY) [ ] as United States Application Number or PCT International

Application Number [ ] and was amended on (MM/DD/YY) [ ] (if applicable).

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is material to patentability as defined in Title 37, Code of Federal Regulations §1.56.

I hereby claim foreign priority benefits under Title 35, United States Code §119(a)-(d) or §365(b) of any foreign application(s) for patent or inventor's certificate or §365(a) of any PCT international application which designated at least one country other than the United States of America, listed below and have also identified below, by checking the box, any foreign application for patent or inventor's certificate, or any PCT international application having a filing date before that of the application on which priority is claimed.

Prior Foreign Application Number(s)	Country	Foreign Filing Date (MM/DD/YY)	Priority Not Claimed	Certified Copy Attached? YES	NO
			[ ]	[ ]	[ ]
			[ ]	[ ]	[ ]
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[ ] Additional foreign applications numbers are listed on a supplemental priority sheet attached hereto:

I hereby claim the benefit under Title 35, United States Code §119(e) of any United States provisional application(s) listed below.

Application Number(s)	Filing Date (MM/DD/YY)	
		[ ] Additional provisional application numbers are listed on a supplemental priority sheet attached hereto.

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QBMAD\176511

Please type a plus sign (+) inside this box [+]

DECLARATION

I hereby claim benefit under Title 35, United States Code §120 of any United States application(s), or §365(C) of any PCT international application designating the United States of America, listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application or PCT international application in the manner provided in the first paragraph of Title 35, United States Code §112, I acknowledge the duty to disclose information which is material to patentability as defined in Title 37, Code of Federal Regulations §1.56 which became available between the filing date of the prior application and the national or PCT international filing date of this application.

U.S. Parent Application Number	PCT Parent Number	Parent Filing Date (MM/DD/YY)	Parent Patent Number *(if applicable)*

[ ] Additional U.S. or PCT international application numbers are listed on a supplemental priority sheet attached hereto

As a named inventor, I hereby appoint the following attorney(s) and/or agent(s) to prosecute this application and all continuation and divisional applications based thereon, and to transact all business in the Patent and Trademark Office connected therewith:

[ ] Firm Name ______ Customer Number or label ______
OR
[X] List attorney(s) and/or agent(s) name and registration number below

Name	Registration Number	Name	Registration Number
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Neil Hamilton	19,869	Keith M. Baxter	31,233
Thomas W. Ehrmann	20,374	John D. Franzini	31,356
Barry E. Sammons	25,608	Joseph W. Bain	34,290
J. Rodman Steele	25,931	Robert J. Sacco	35,667
Nicholas J. Seay	27,386	Jean C. Baker	35,433
George E. Haas	27,642	David G. Ryser	35,407
Michael J. McGovern	28,326	Bennett J. Berson	37,094
Carl R. Schwartz	29,437	Michael A. Jaskolski	37,551

[ ] Additional attorney(s) and/or agents named on a supplemental priority sheet attached hereto

Please direct all correspondence to [ ] Customer Number or label ______ OR [X] Fill in correspondence address below

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Address	Quarles & Brady LLP				
Address	P O Box 2113				
City	Madison	**State**	WI	**Zip**	53701-2113
Country	US	**Telephone**	608/251-5000	**Fax**	608/251-9166

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Name of Sole or First Inventor: [ ] A petition has been filed for this unsigned inventor

Given Name	Ronald	Middle Initial	T.	Family Name	Raines	Suffix e.g. Jr.	
Inventor's Signature	Ronald T. Raines					Date	1/4/99
Residence: City	Madison	State	WI	Country	US	Citizenship	US
Post Office Address	2320 Lakeland Avenue						
Post Office Address							
City	Madison	State	WI	Zip	53704	Country	US

Applicant Authority [ ]

[ ] Additional inventors are being named on supplemental sheet(s) attached hereto